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(57) Abstract

A method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of: (i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium an analyte receptor capable of binding to the target analyte or a predetermined amount of analyte; (ii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element an analyte detection agent which detects the presence of target analyte in the test sample, said analyte detection agent having a label associated therewith; (iii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element a calibration agent having a label associated therewith; (iv) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent; (v) contacting the lateral flow liquid permeable medium with the test sample; and (vi) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

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TITLE

"METHOD AND APPARATUS FOR QUANTITATIVE AND SEMI-QUANTITATIVE DETERMINATION OF AN ANALYTE"

FIELD OF THE INVENTION

THIS INVENTION relates generally to analyte assays. In particular, this invention relates to an assay and apparatus for semi-quantitative determination of an analyte in a test sample. The invention also relates to quantitative determination of an analyte by use of an apparatus which reads and processes the results of the assay of the invention.

BACKGROUND OF THE INVENTION

In the medical, chemistry, environmental testing and veterinary fields there is a need to measure an ever increasing number of substances in physiological, biological or test samples. Methods which have been used for the detection of these compounds vary widely but often involve the use of expensive and sophisticated equipment or cumbersome multi-step procedures which require considerable training and care. These requirements restrict the general diagnostic utility of many methods.

Numerous attempts have been made in the past to simplify such methods so that measurement of a substance of diagnostic importance can be carried out at convenient locations including point of care locations such as clinics, medical practitioners' offices, veterinary surgeries, homes or generally any on site location without the need for expensive equipment or training.

Generally, most of the assay systems that have been developed for these purposes facilitate qualitative determination of specific analytes in a test sample but do not allow estimation of the level of an analyte with any degree of confidence. Indeed, for many disease states, it is the measured level rather than the simple presence or absence of a particular analyte which is of primary diagnostic significance. For instance, in cases where changes in the concentration of an analyte are used to

monitor a disease course, it would be advantageous to determine by visual or instrumental inspection whether the levels of the analyte were increasing or decreasing.

One attempt at developing such an assay is disclosed in U.S. Patent Specification 5,073,484 which is directed to a method and an apparatus for the quantification of an analyte in a liquid employing a liquid-permeable solid medium defining a liquid flow path. The medium includes a number of reaction zones, containing a reactant, spaced apart along the flow path and in which reaction occurs between the reactant and the analyte or an analyte derivative (e.g. a labelled analyte) to result in the formation of a predetermined product. Detector means are employed to detect analyte, analyte derivatives, reactant or predetermined product in the reaction zones, the number of such zones in which such detection occurs indicating the amount of analyte in the liquid.

The method is predicated on the condition that for a given amount of analyte in a liquid, the analyte must be bound substantially to saturation in a first reaction zone before any residual amount of the analyte is bound subsequently in a second reaction zone and so on. Hence, this method provides theoretically for a linear relationship between the number of reaction zones in which detection occurs and the amount of analyte in a liquid.

Recent analysis of this method by the applicants, however, has revealed that when colloidal gold is used as the detector means in concert with a flow membrane, the amount of analyte in a liquid is not directly proportional to the number of reaction zones in which detection occurs. The results of this analysis, illustrated in FIG.1, indicate that when a flow membrane comprising five reaction zones is used for the quantitation of an analyte, all five reaction zones detect the analyte independently of the analyte concentration. In addition, the intensities of all reaction zones are equivalent and are directly proportional to the amount of analyte in the liquid.

For example, when a relatively low amount of analyte is applied, the intensities of all five reaction zones are equivalently low. Conversely, when a relatively high amount of analyte is applied, the intensities of all five reaction zones are equivalently high. To this end, it is believed that it is not possible to reduce this method to practice insofar as using a flow membrane and colloidal gold as the detector means are concerned.

It also will be appreciated that US Patent 5,073,484 in including a multiplicity of reaction zones does not include a calibration zone which has a calibration agent receptor bound thereto.

A further attempt at developing a semi-quantitative assay is disclosed in Australian Patent Specification 658566. One embodiment of this assay utilizes a vertical flow-through filter comprising a test zone and one or more calibration zones. At the test zone there is non-diffusibly attached therein an analyte receptor capable of binding a target analyte, and at the or each calibration zone there is non-diffusibly attached therein a calibration receptor capable of binding a calibration agent. When there is more than one calibration zone, one of these zones is capable of binding a higher predetermined amount of the calibration agent than the other.

In operation, a test sample suspected of containing the target analyte is introduced to the filter and the target analyte, if present, binds to the analyte receptor at the test zone. A solution comprising a labeled analyte detection agent as well as a labeled calibration agent is added subsequently to the filter. The analyte detection agent binds to the target analyte at the test zone and the calibration agent binds to the calibration receptor at the calibration zone in amounts which are directly proportional to the amounts of target analyte and calibration receptor bound to the filter. The presence of analyte detection agent bound at the test zone and the presence of calibration agent bound at the or each calibration zone is determined subsequently by signal development, permitting qualitative or semi-quantitative determination of the target analyte in the test sample.

In an alternative embodiment, the target analyte in the test sample is premixed with a solution comprising labeled analyte detection agent and labeled calibration agent prior to introduction of the mixture onto the filter.

Although the above assay provides improvement in facilitating semi-quantitative determination of an analyte in a test sample, there are a number of disadvantages. It is necessary, for example, to remove unbound analyte detection agent and unbound calibration agent from the filter and this has to be achieved by incubation and washing steps as described in specification AU 658566. Such incubation and washing steps in the case of unbound analyte detection agent and unbound calibration agent must take place before a meaningful comparison of the respective signals in the test and calibration zones may be made. Accordingly, the analyte assay of AU 658566 is cumbersome and relatively time-consuming for effective operation which will therefore limit the convenience or utility thereof in instances when analyte assays are required to be performed with rapidity such as, for example, in medical surgeries.

Having regard to foregoing, there is at present a paucity of assay systems either described or commercially available which can provide simple, rapid semi-quantitative and/or quantitative determination of a target analyte.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a simple method for semi-quantitative and/or quantitative determination of an analyte in a test sample which substantially reduces the number of steps required to perform an analyte assay.

It is a further object of the invention to provide an apparatus for use in concert with the method of the invention.

According to one aspect of the invention, there is provided a method for quantitative or semi-quantitative determination of target analyte(s)

in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium an analyte receptor capable of binding to the target analyte;

(ii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element an analyte detection agent which detects the target analyte if present in the test sample, said analyte detection agent having a label associated therewith;

(iii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element a calibration agent having the same label associated therewith;

(iv) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(v) contacting the lateral flow liquid permeable medium with the test sample; and

(vi) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

The target analyte(s) may be selected from a group including an antigenic substance, a hapten, an antibody, a protein, a peptide, an amino acid, a nucleic acid, a hormone, a steroid, a vitamin, a carbohydrate, a lipid, a blood clotting factor, a pathogenic organism for which polyclonal and/or monoclonal antibodies can be produced, a natural or synthetic chemical substance, a contaminant, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, and metabolites of or antibodies to any of the above substances.

It of course will be appreciated that the number of test zones utilized in the lateral flow liquid permeable medium will be dependent on the

number of target analytes being quantified. In the preferred embodiment, however, the lateral flow liquid permeable medium will only include a single test zone for quantification of a single target analyte.

Preferably, the target analyte has two binding sites each of which is capable of forming a specific binding pair with a specific binding partner.

A specific binding pair, as used herein, comprises two different molecules wherein one of the molecules through chemical or physical means specifically binds to a second molecule. Such specific binding partners, examples of which are described in U.S. Patent Specification 5,075,078, include antigens and antibodies, lectins and carbohydrates, complementary peptides, protein, carbohydrate and nucleic acid structures, enzyme inhibitors and enzymes, Protein A and IgG as well as effector and receptor molecules.

It will of course be appreciated that it is sufficient for the target analyte to have a single binding site for a specific binding partner if the target analyte in combination with a first specific binding partner is capable of producing a unique binding site for a second specific binding partner.

The test sample is preferably an aqueous solution.

The term "test sample" as used herein may include a biological fluid which may be extracted, untreated, treated, diluted or concentrated from a plant or animal.

Preferably, the biological fluid is derived from an animal.

Suitably, the biological fluid is selected from a group including whole blood, serum, plasma, saliva, urine, sweat, ascitic fluid, peritoneal fluid, synovial fluid, amniotic fluid, cerebrospinal fluid and the like.

Preferably, the calibration agent and the analyte detection agent are diffusibly attached to the separate support element rather than the lateral flow liquid permeable medium. In this regard, the separate support element is in fluid communication with the lateral flow liquid permeable

medium such that the test sample may pass or migrate from the separate support element to the lateral flow liquid permeable medium. Fluid communication may include physical contact of the separate support element to the lateral flow liquid permeable medium as well as the separation of the lateral flow liquid permeable medium by an intervening space or additional material which still allows fluid communication between the element and the liquid permeable medium.

Preferably, the separate support element partially overlaps and is in close proximity to an end portion of the lateral flow liquid permeable medium. Alternatively, the separate support element may form part of one end of the lateral flow liquid permeable medium.

The separate support element may be composed of any suitable material which can transfer the test sample to the lateral flow liquid permeable medium.

Preferably, the separate support element is adapted to absorb a volume of test sample which is equal to or greater than the total volume capacity of the lateral flow liquid permeable medium.

A material suitable for use as the separate support element may include nitrocellulose, cellulosic paper, porous polyethylene frit or pads and glass fibre filter paper.

The separate support element may contain one or more assay reagents either diffusibly or non-diffusibly attached therein.

Preferably, the assay reagents in the separate support element are diffusibly attached therein.

Suitable assay reagents that may be contained in the separate support element include the analyte detection agent and the calibration agent.

Preferably, the analyte detection agent is a specific binding partner of the target analyte which may have an associated label.

Alternatively, in another embodiment of the invention, the

analyte detection agent may be a predetermined amount of the target analyte itself having a label associated therewith. In this embodiment, the analyte, which is preferably directly labeled, acts as the analyte detection agent by competing with the analyte in the test sample for binding to a specific binding partner which is non-diffusibly attached in the test zone.

It will of course be appreciated that in such an embodiment, an analog of the analyte may also be used as the analyte detection agent. The term "analog" as used herein refers to a compound which for the purposes of a particular assay behaves substantially the same as the analyte.

The analyte detection agent and the calibration agent each have a label associated therewith which includes the following:

i. direct attachment of the label to the analyte detection agent;

ii. indirect attachment of the label to the analyte detection agent; i.e., attachment of the label to another assay reagent which subsequently binds to the analyte detection agent; and

iii. attachment to a subsequent reaction product of the analyte detection agent.

In a similar manner, the calibration agent has the same label associated therewith which also may include i., ii., and iii. above.

Preferably, the label is attached directly to the analyte detection agent and the calibration agent.

The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu^{34}), a radioisotope and a direct visual label.

In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

A large number of enzymes suitable for use as labels is

disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338, all of which are herein incorporated by reference. Suitable enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme which is in solution.

Suitably, the fluorophore is selected from a group including fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITL) or R-Phycoerythrin (RPE).

Preferably, the label is a colloidal metallic particle.

Most preferably, the label is colloidal gold.

The calibration agent is capable of forming a specific binding pair with a specific binding partner.

Preferably, the calibration agent has a high affinity for the specific binding partner thereof.

The calibration agent is most suitably avidin, streptavidin or biotin or other calibration agent which has high affinity for the specific binding partner thereof.

The passage of the mixture through the lateral flow liquid permeable medium may be effected by capillary migration, gravity flow or electrophoresis or a medium of hydrophilic nature having an affinity for water.

Preferably, the passage of the mixture is effected by capillary migration.

The lateral flow liquid permeable medium may be any suitable absorbent, porous, capillarity-possessing or bibulous material through which a solution containing the target analyte can be transported.

Natural, synthetic or naturally occurring materials which are synthetically modified may be used as the liquid permeable medium including but not limited to cellulose materials such as paper, cellulose and

cellulose derivatives such as cellulose acetate and nitrocellulose; glass fibres; natural or synthetic cloth; porous gels such as agarose, silica gel, dextran and gelatin; porous fibrous matrixes; starch based materials such as Sephadex® cross-linked dextran chains; ceramic materials; films of polyvinyl chloride and polyamide; and combinations of polyvinyl chloride-silica and the like.

Preferably, the lateral flow liquid permeable medium is a cellulose material.

More preferably, the lateral flow liquid permeable medium is nitrocellulose.

Suitably, the lateral flow liquid permeable medium does not interfere with the production of the detectable signal.

The method of the invention may also include an additional step of filtering the test sample.

The filtering may be effected by any filter or trapping device used to remove particles above a certain size from the test sample. For example, the filtering step may be used to remove red blood cells from a sample of whole blood, such that plasma is the fluid which is received by the separate support element and transferred to the lateral flow liquid permeable medium.

Preferably, the specific binding partner of the analyte receptor is the analyte or analog thereof.

Alternatively, in a further embodiment of the invention, the specific binding partner of the analyte receptor may be the analyte detection agent. In such a case, the analyte detection agent may be a predetermined amount of analyte having a label associated therewith.

The at least one calibration zone comprises a predetermined amount of a calibration agent receptor non-diffusibly attached therein which is capable of participating in the formation of an immobilized specific binding pair with the calibration agent.

The predetermined amount of the calibration agent receptor is selected so that a substantially finite amount of the calibration agent is bound in the at least one calibration zone.

Preferably, the predetermined amount is such that when the substantially finite amount of the calibration agent is bound in the calibration zone, the signal generated therein is within a dynamic range suitable for comparing with the signal generated in the test zone.

In preference, there are at least two calibration zones. In such an example, each calibration zone has a different predetermined amount of the calibration agent receptor so that, in operation, each calibration zone produces a different signal.

Non-diffusive attachment of the analyte receptor and the calibration agent receptor may be effected by any suitable means including covalent bonding, non-covalent bonding or entrapment.

It will of course be appreciated that the receptors used in the invention may be non-diffusibly attached either directly or indirectly. For example, each receptor may be covalently bound to a spacer molecule which has been covalently bound to the lateral flow liquid permeable medium.

The spacer molecule may include a latex microparticle, a protein such as bovine serum albumin (BSA) or a polymer such as dextran.

The signals generated in the test zone and the at least one calibration zone may be measured by visual inspection or by an instrumental means.

Preferably, the signals are measured by the instrumental means. In such a case, the signal is preferably a colored signal and the preferred method of measurement is reflectance.

Measurement of the signals may be made by any instrument which is capable of converting signal into a digital expression that is proportional to the signal produced.

Depending on the nature of the label, a signal may be

instrumentally detected by irradiating a fluorescent label with light and measuring the level of fluorescence in a fluorimeter; by providing for an enzyme system to produce a dye which could be measured using a spectrophotometer; or detection of a dye particle or a colored colloidal metallic or non metallic particle using a reflectometer; in the case of using a radioactive label or chemiluminescent molecule employing a radiation counter.

In this invention, comparison of the signals generated in at least one test zone and the at least one calibration zone is used to determine the approximate concentration of the analyte in the test sample. This comparison is predicated on the condition that a signal correlates with a certain amount or concentration of a label captured in a zone which in turn corresponds proportionally to a specific amount of the analyte. Thus, a predetermined amount of a calibration agent receptor in a calibration zone is chosen to bind substantially a finite amount of a calibration agent which in turn must be bound by a certain amount of the label and the signal generated therefrom must be reflective of a specific amount of the analyte.

In one possible situation there may be provided a multiplicity of test zones for determination of multiple target analytes. In this situation there will only be a single calibration zones.

The method of the invention further includes within its scope the use of one or more calibration agents in step (iii) wherein there is also provided one or more complementary calibration agent receptors each of which is non-diffusibly attached respectively in different calibration zones.

The method of the invention still further includes within its scope the use of a procedural control step which indicates validation of the methods of the invention.

The procedural control step may be effected by inclusion of a procedural control zone which may form part of the lateral flow liquid permeable medium.

The completion of the method may be indicated by a changing of color which may be effected by an assay reagent contacting the procedural control zone.

Preferably, the assay reagent is the analyte detection agent having a label associated therewith.

Alternatively, the assay reagent may be a compound which changes color upon contact with a test solution containing water which compound includes a dehydrated transition metal salt such as $CuSO_4$, $Co(NO_3)_2$, and the like or a pH indicator which responds to a pH of a buffered reagent solution.

In a second aspect of the invention, there is provided a method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium a predetermined amount of analyte;

(ii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element an analyte detection agent which detects the target analyte if present in the test sample, said analyte detection agent having a label associated therewith;

(iii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element a calibration agent having the same label associated therewith;

(iv) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(v) contacting the lateral flow liquid permeable medium with the test sample; and

(vi) comparing signals associated with each label at the test and calibration zone(s) to effect determination of the target analyte in the test

sample.

In this alternative embodiment, the analyte detection agent will comprise a specific binding partner of the target analyte which may have the same label associated therewith as the calibration agent.

According to a further aspect of the invention there is provided a lateral flow liquid permeable medium adapted to convey fluids at least one fluid flow direction for use in the method according to the first aspect of the invention comprising:

(1) at least one test zone having non-diffusibly attached therein an analyte receptor which is capable of participating in the formation of an immobilized specific binding pair with an analyte or analyte detection agent;

(2) at least one calibration zone having non-diffusibly attached therein a predetermined amount of a calibration agent receptor which is capable of participating in the formation of an immobilized specific binding pair with a calibration agent; and

(3) an assay reagent zone including a calibration agent having a label associated therewith and the analyte detection agent which has the same label associated therewith.

According to a still further aspect of the invention there is provided a lateral flow liquid permeable medium adapted to convey fluids in at least one fluid flow direction for use in the method according to the second aspect of the invention comprising:

(1) at least one test zone having non-diffusibly attached therein a predetermined amount of analyte which is capable of participating in the formation of an immobilized specific binding pair with an analyte detection agent;

(2) at least one calibration zone having non-diffusibly attached therein a predetermined amount of a calibration agent receptor which is capable of participating in the formation of an immobilized specific

binding pair with a calibration agent; and

(3) an assay reagent zone including a calibration agent having a label associated therewith and the analyte detection agent which has the same label associated therewith.

Preferably, the lateral flow liquid permeable mediums described above further include a separate support element which has diffusibly attached thereon the calibration agent and associated label and the analyte detection agent and associated label.

The lateral flow liquid permeable medium preferably includes a filter in fluid communication with said separate support element.

Suitably, the lateral flow liquid permeable medium includes a procedural control zone as described above.

The liquid permeable medium may also include an absorbent pad which is downstream of said test and said at least one calibration zone, which absorbent pad is adapted to absorb an excess amount of a test sample or a reagent solution.

The liquid permeable medium may include in one situation a forward flow and reverse flow of sample as well as diffusible reagents. However, most preferrably the liquid permeable medium is designed to accommodate only a single fluid flow direction.

The invention also comprises a kit for use in the methods as herein described including the lateral flow liquid permeable medium as described above. Such kit may incorporate a housing which incorporates the lateral flow liquid permeable medium and optionally the separate support medium as described above with appropriate windows for viewing the test zone and calibration zone and optionally a window for viewing the procedural control zone described above. The housing may also include an aperture for introduction of the test sample suspected of containing the target analyte.

The invention may also include a signal measuring device which includes the housing as described above. Suitably the signal

measuring device measures the signals as described above and is programmed to contain a multiplicity of standard curves for the various analytes which may be obtained from the calibration zones as for example described in an article by Diebold E, *et al.*, Chemtech 1991; **462-7** and an article by Burke A, *et al.*, Chemtech 1991; **547-551** which are hereby incorporated by reference.

A lysis solution may also be provided suitable for lysis of red blood cells in the test sample.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by example with reference to the following drawings in which:

FIG. 1 illustrates the results of two analyte assays utilizing lateral flow membranes constructed in accordance with U.S. 5,073,484 and colloidal gold as the detector means wherein the top illustration refers to a an assay result in which a relatively low amount of target analyte was assayed and the bottom illustration refers to an assay result in which a relatively high amount of target analyte was assayed;

FIG. 2 refers to a side view illustration of one embodiment of an unused lateral flow liquid permeable medium constructed in accordance with the invention.

FIG. 3 refers to a top plan view of five lateral flow liquid permeable mediums constructed in accordance with FIG. 2 wherein each is contained within a cartridge and wherein different assay results are shown.

FIG. 4 refers to a side view illustration of the lateral flow liquid permeable medium of FIG. 2 after completion of an assay in which no target analyte was present in the test sample.

FIG. 5 refers to a side view illustration of the lateral flow liquid permeable medium of FIG. 2 after completion of an assay in which target analyte was present in the test sample.

FIG. 6 refers to a box plot illustrating a comparison of the visual semi-quantitative determination of D-dimer with a quantitative determination of D-dimer by EIA.

FIG. 7 refers to a box plot illustrating a comparison of the visual semi-quantitative determination of myoglobin with a quantitative determination of myoglobin by EIA.

FIG. 8 refers to a side view illustration of an alternative embodiment of an unused lateral flow liquid permeable medium constructed in accordance with the invention.

FIG. 9 refers to a top plan view of four lateral flow liquid permeable mediums constructed in accordance with FIG. 8 wherein each is contained within a cartridge and wherein different assay results are shown.

FIG. 10 refers to a side view illustration of the lateral flow liquid permeable medium of FIG. 8 after completion of an assay in which no target analyte was present in the test sample.

FIG. 11 refers to a side view illustration of the lateral flow liquid permeable medium of FIG. 8 after completion of an assay in which no target analyte was present in the test sample.

FIG. 12 refers to a linear regression analysis corresponding to a comparison of quantitative determination of D-dimer by instrument with quantitative determination of D-dimer by EIA.

FIG. 13 refers to a linear regression analysis corresponding to a comparison of quantitative determination of myoglobin by instrument with quantitative determination of myoglobin by EIA.

FIG.14 refers to a standard curve of reflectance signal as a function of biotin - BSA concentration.

EXAMPLE 1

Semi-quantitative determination of fibrin D-dimer

A side view diagrammatic representation of a device comprising the lateral flow liquid permeable medium constructed in

accordance with the invention is shown in FIG. 2. The device consists of a filter 201 adapted to remove red blood cells from a test sample, a nitrocellulose membrane 207 and a separate support element 202 containing a suitable concentration of reagents including the D-dimer binding monoclonal antibody DD-3B6/22 labeled with colloidal gold (3B6-gold; *i.e.*, analyte detection agent) 208 specific for the thrombosis marker D-dimer 213 (*i.e.*, target analyte) under test and streptavidin labeled with colloidal gold (streptavidin-gold; *i.e.*, calibration agent) 209.

The separate support element 202 receives the test sample, and the wetting of the element by the test sample performs at least two functions. Firstly, it dissolves or reconstitutes a predetermined amount of 3B6-gold 208 and streptavidin-gold 209. Secondly, it initiates the transfer of both the test sample containing D-dimer 213 and the freshly dissolved reagents 208, 209. Both the 3B6-gold and the streptavidin-gold are mobilized when moistened by the test sample and/or an application buffer.

The nitrocellulose membrane 207 comprises a test zone 204 and two calibration zones 210, 211. Non-diffusibly attached at a first calibration zone 210 is a first predetermined amount of BSA labeled with biotin (BSA-biotin; *i.e.*, calibration agent receptor) 214 capable of binding specifically to the streptavidin-gold. At the second calibration zone 211 there is non-diffusibly attached a second predetermined amount of BSA-biotin 216. The concentration of BSA-biotin at the second calibration zone 211 is adjusted to generate a different signal intensity compared to that generated at the first calibration zone 210. In addition, there is non-diffusibly attached at the test zone 204 a predetermined amount of a monoclonal antibody termed DD-1D2/48 1D2 antibody; (*i.e.*, analyte receptor) 215 capable of binding D-dimer when complexed to the 3B6-gold.

Also included is a procedural control zone 212 containing an anti-mouse antibody 217 which is non-diffusibly attached therein and is capable of specifically binding the 3B6-gold.

At the end of the membrane 207 there is provided an absorbent pad 206 to absorb an excess of test sample, application buffer or assay reagents.

For some samples, *e.g.* plasma, serum, or urine, the filter 201 which is adapted to remove red blood cells or particulate matter may not be necessary.

In operation, addition of the test sample and/or sample application buffer causes moistening of the separate support element which subsequently mobilizes the 3B6-gold 208 and streptavidin-gold 209. If D-dimer 213 is present in the test sample, it binds the 3B6-gold 208 via a first antigenic site to form a complex. This complex, in concert with the streptavidin-gold 209, subsequently migrates to the first calibration zone 210 whereat a substantially finite amount of the streptavidin-gold binds to the first predetermined amount of BSA-biotin 214 in the first calibration zone 210. The analyte-3B6-gold complex subsequently migrates to the test zone 204 whereat the complex binds the 1D2 antibody 215 via a second antigenic site of D-dimer 213. Streptavidin-gold 209 which has not bound to the first calibration zone 210 subsequently migrates to the second calibration zone 211 whereat it binds to a second predetermined amount of BSA-biotin 216.

The concentrations of the streptavidin-gold 209 and the BSA-biotin 214, 216 are adjusted by methods described in Example 6 to give signal intensities that fall within a suitable dynamic range of those produced by typical diagnostic concentrations of D-dimer in blood or plasma to generate calibration zones which, when in operation, generate signal intensities corresponding to 120 ng/mL and 1000 ng/mL of D-dimer. The introduction of such calibration agents enable visual inspection of a test device to assign one of three values to any test sample (FIG.3).

With reference to FIG. 3, it should be noted that the device as illustrated in FIG. 2 is now contained within a housing or cartridge comprising a sample receiving aperture (S) which is in communication with the separate

support element. The housing also includes a window which facilitates viewing of the procedural control (C), test (T), as well as the 120 ng/mL and 1000 ng/mL calibration zones.

Now turning to FIG. 3a, there is shown a top view of an unused device for semi-quantitative measurement of human D-dimer. Note that there are no visible or instrumental read lines in the viewing window.

With reference to FIG. 3b, there is shown the test device after the successful completion of a negative test. Note the appearance of zones of color in the viewing window at the 120 ng/mL and 1000 ng/mL calibration zones and the procedural control zone and the absence of any color at the test zone. This allows a value of less than 120 ng/mL of D-dimer to be assigned to the test sample.

In relation to FIG. 3c, there is shown the device after the successful completion of a test with a sample containing less than 120 ng/mL D-dimer. In this case, there is a visible line at the test zone but of lower intensity than the 120 ng/mL calibration zone thus allowing a value of less 120 ng/mL of D-dimer to be assigned to this test sample. The respective intensities of the other zones are similar to those in FIG. 3b.

With reference to FIG. 3d, there is shown the device after the successful completion of a test on a sample containing a D-dimer concentration of between 120 ng/mL and 1000 ng/mL. Note the zone of intensity in the test zone is now intermediate between the 120 ng/mL and 1000 ng/mL calibration zones allowing an intermediate value to be assigned to the test sample.

Finally, FIG. 3e shows the device after the successful completion of a test on a sample containing more than 1000 ng/mL D-dimer. Note, that at the test zone there is a signal intensity greater than that of the 1000 ng/mL calibration zone.

Also, in this system, any unbound labeled reagents migrate to the absorbent pad where they are not visible to the observer. For a

summary of the aforementioned results see Table 1.

FIG. 4 is a side view diagrammatic representation of the assay reagents after completion of a test in which no D-dimer was present in the sample. The labeled reagents 208, 209 have migrated from the separate support element 202 and the streptavidin-gold 209 has bound to the calibration zones 210, 211. The 3B6-gold 208 has passed through the test zone 204 without significant binding and consequently, there was no visible or instrument determinable signal at the test zone 204.

FIG. 5 is a side view diagrammatic representation of a completed test in which a significant concentration of D-dimer 213 was present in the test sample. In this case, not only has the streptavidin-gold 209 been captured at the respective calibration zones 210, 211 but the analyte-3B6-gold complex has also been captured at the test zone 204.

An estimation of the concentration of the D-dimer 213 in the test sample is made by comparing the signal intensity generated in the test zone 204 with those generated in the respective calibration zones 210, 211.

A comparison of the visual semi-quantitative determination of D-dimer with a quantitative determination of D-dimer by enzyme immunoassay (EIA) (DIMER TEST EIA, Agen Biomedical, Australia) is shown in FIG. 6. The data is presented as a box plot. The visual semi-quantitative determination was made with 187 plasma samples of clinical origin using 25 µL of sample. At 10 minutes after the tests were run the visual intensity of the test zone was compared with the visual intensity of the two calibration zones. The results show that the 102 samples graded visually to be less than the 120 ng/ml had a median value of 46 ng/ml when measured by EIA, 75% were less than 80 ng/ml and 90% were less than 150 ng/ml. Samples graded visually to be between 120 ng/ml and 1000 ng/ml usually had values in this range. The higher the D-dimer level in the sample the more likely a high visual grading.

EXAMPLE 2

Semi-quantitative determination of myoglobin using specific monoclonal antibodies directed against myoglobin

The test is performed on serum, plasma, anti-coagulated whole blood samples (citrate, EDTA, heparin) or finger stick samples directly applied to the test device using the following procedure.

Test Procedure

The components are allowed to come to room temperature prior to use, keeping a pouch containing the test devices sealed to avoid condensation on the test surface. The pouch is then opened and the test devices placed on a flat horizontal surface.

Fifty microlitres of blood or 25 µL of plasma or serum is added to the sample well labeled (S) followed by 2 x 50 µL of saline (0.9% NaCl). The sample is then allowed to migrate for ten minutes after which the respective intensities of a color at the test and two calibration zones are compared. In this case, the device comprises a first calibration zone corresponding to a value of 70 ng/mL myoglobin, a second calibration zone corresponding to a value of 350 ng/mL myoglobin and a test zone therebetween.

The test is valid if, after 10 minutes, a color appears in the 70 ng/mL and 350 ng/mL calibration zones with the condition that the color corresponding to the 350 ng/mL calibration zone is more intense compared to the color corresponding to the 70 ng/mL calibration zone.

Samples with myoglobin values less than 70 ng/mL at the test zone (T) have either no color in the test zone or a color of equal intensity or less compared to that in the 70 ng/mL calibration zone.

Samples with myoglobin levels higher than 70 ng/mL may be of clinical significance and result in a color in the test zone of intensity greater than that in the 70 ng/mL calibration zone. If the colour in the test zone is less intense than that in the 350 ng/mL calibration zone, then the

sample has a value less than 350 ng/mL.

A comparison of the visual semi-quantitative determination of myoglobin with a quantitative determination of myoglobin by EIA (Stratus EIA, DADE, USA) is shown in FIG. 7. The data is presented as a box plot. The visual semi-quantitative determination was made with 127 plasma samples of clinical origin using 25 µL of sample. At ten minutes after the tests were run the visual intensity of the test zone was compared to the visual intensity of the two calibration zones. The results show that the 59 samples graded visually to be less than the 70 ng/ml had a median value of 47 ng when measured by EIA, 75% were less than 70 ng/ml and 90% were less than 85 ng/ml. Samples graded visually to be between 70 ng/ml and 350 ng/ml usually had values in this range. The higher the myoglobin level in the sample the more likely a high visual grading.

EXAMPLE 3

Semi-quantitative determination of digoxin by competition assay

The device can also be configured to allow the semi-quantitation of smaller analytes such as drugs, including therapeutic drugs and illicit drugs, hormones, vitamins, steroids, peptides and haptens as well as the larger molecular weight molecules which can be assayed in the sandwich format outlined in Examples 1 and 2.

A side view diagrammatic representation of a competition assay in accordance with the invention is shown in FIG. 8. The device consists of a filter 601 adapted to remove red blood cells, a separate support element 602 containing a suitable concentration of reagents including anti-digoxin gold-labeled antibody 603 (*i.e.*, analyte detection agent) specific for the drug digoxin 611 (*i.e.* target analyte) and a streptavidin-gold 604 calibration agent.

As described in Example 1, the test device comprises a nitrocellulose membrane 605 upon which is non-diffusibly attached at first

and second calibration zones 606, 608 different predetermined amounts 614, 615 of BSA-biotin 604 which when in operation generate different signal intensities (FIG. 8).

In addition, the membrane 605 includes a test zone 607 whereat there is non-diffusibly attached an unlabeled digoxin (or an unlabeled analog thereof) 613 which is capable of binding specifically to the anti-digoxin gold-labeled antibody 603.

Alternatively, in the case wherein the separate support element contains a labeled digoxin as the detection agent, the test zone has non-diffusibly attached therein an unlabeled anti-digoxin antibody which is capable of binding specifically the labeled digoxin and unlabeled digoxin if present in the test sample.

As described in the previous example, the membrane also includes a procedural control zone 609 which has non-diffusibly attached therein an anti-mouse antibody 616 capable of binding the anti-digoxin gold-labeled antibody 603 which has not bound to the test zone 607. Adjacent to one end of the membrane 605, there is also provided an absorbent pad 610 which absorbs an excess amount of the test sample, the application buffer or assay reagents.

It will also be appreciated that when labeled digoxin is employed as the analyte detection agent in, for example a competitive assay, an anti-digoxin antibody may be used as a capturing component in the procedural control zone.

In operation, addition of the test sample and/or the application buffer causes moistening of the separate support element 602 and subsequent mobilization of the anti-digoxin gold-labeled antibody 603 and the streptavidin-gold 604. If digoxin 611 is present in the test sample, it binds and complexes with the anti-digoxin gold-labeled antibody 603 and, in concert with the streptavidin-gold 604, migrates subsequently to a detection area comprising the test zone 607 and calibration zones 606, 608.

In the test zone 607, the binding of anti-digoxin gold-labeled antibody 603 to the attached unlabeled digoxin 613 is inhibited in the presence of digoxin 611. This results in a test zone 607 of reduced intensity compared to that generated in the absence of digoxin 611. When digoxin 611 is absent in the test sample, a zone of strong intensity is generated in the test zone 607.

In the case of the separate support element containing labeled digoxin or analog thereof, the digoxin, if present in the test sample, and the labeled digoxin migrate along the membrane without significant interaction. In the test zone, the binding of labeled digoxin to non-diffusibly attached unlabeled anti-digoxin antibody is inhibited in the presence of digoxin.

The concentrations of the streptavidin-gold 604 and BSA-biotin 612 are adjusted to give signal intensities that fall within a suitable dynamic range of those produced by typical therapeutic concentrations of digoxin 611 as described in Example 6.

For the measurement of the digoxin in blood or plasma, the lower calibration zone, when in use, generates signal intensities corresponding to approximately 0.8 ng/mL of digoxin. The limit of detection of this analyte in a flow through device is 0.4 ng/mL and consequently, the use of calibration zones generating signal intensities corresponding to 0.8 ng/mL and 1.6 ng/mL respectively enable visual inspection of a test device to assign one of four values to a given sample (FIG. 9).

With reference to FIG. 9, it should be noted that the device according to FIG. 8 is now contained within a housing or cartridge as described in Example 1.

In relation to FIG. 9a, there is shown a top view of an unused device for semi-quantitative measurement of digoxin. Notice that there are no visible instrumental read lines in the viewing window comprising procedural control (C), test (T), 0.8 ng/mL and 1.6 ng/mL calibration zones.

Now turning to FIG. 9b, there is shown the test device after the

successful completion of a test not containing sub-therapeutic doses of digoxin. Note the appearance of zones of color in the 0.8 ng/mL and 1.6 ng/mL calibration zones and the procedural control zone and the intense color generated in the test zone. This allows a value of < 0.8 ng/mL digoxin to be assigned to this test sample.

With reference to FIG. 9c, there is shown the device after the successful completion of test with a sample containing between 0.8 ng/mL to 1.6 ng/mL digoxin. In this case, a visible line results in the test zone and is of lower intensity than that generated in the 0.8 ng/mL calibration zone but stronger than that generated in the 1.6 ng/mL calibration zone allowing a value of between 0.8-1.6 ng/mL of digoxin (the normal therapeutic range) to be assigned to this sample. The signal intensities of the other zones are similar to those shown in FIG. 9b.

With reference to FIG. 9d , there is shown the device after the successful completion of a test on a sample containing a digoxin concentration of > 1.6 ng/mL (toxic range). Note the zone of intensity in the viewing window; the test zone has generated a signal intensity lower than that generated in the 1.6 ng/mL calibration zones allowing a higher value to be assigned to the sample. Any unbound labeled reagents migrate to the absorbent pad where they are not visible to the observer. These results are summarized in Table 2.

FIG. 10 shows a side view diagrammatic representation of the assay reagents after completion of a test in which no digoxin was present in the test sample. The labeled reagents 603, 604 have migrated from the separate support element 602 and the streptavidin-gold has bound to the BSA-biotin 612 in the calibration zones 606, 608. The anti-digoxin gold-labeled antibody 603 has bound to the attached unlabeled digoxin 613 in the test zone 607 generating a maximal signal.

FIG. 11 shows a completed test in which toxic concentrations of digoxin 611 were present in the test sample. In this case the streptavidin-

gold has been captured at calibration zones 606, 608 but the digoxin 611 has inhibited the binding of the anti-digoxin gold-labeled antibody 603 at the test zone generating a reduced signal compared to that generated in the absence of digoxin 611 in the test sample.

EXAMPLE 4

Quantitative determination of analyte concentration by instrument reading of test devices.

The lateral flow liquid permeable medium in accordance with the invention, which is preferably contained within a housing or cartridge, may be read visually after completion of an assay, as described in Examples 1 to 3. Alternatively, the lateral flow liquid permeable medium can be read in an instrument configured to receive the device and to measure the change in optical reflectivity produced by the attachment of the label onto the surface of the membrane. The instrument is preferably configured to measure reflectance at a wavelength corresponding to the appropriate, usually maximum, absorbance of the label. It can include more than one LED or suitable light source for measuring and detecting a multiple of reflected wavelengths. The instrument is programmed to contain standard curves for the various tests to be read and each test has a code, interpreted by the instrument, such that the correct data for a specific target analyte is accessed.

In designing a test for instrumental reading, it is not necessary to position the calibration zones such that the density of the band is seen to conform to the density of a test reading at clinically relevant levels, such as the top of a normal range of such levels. It is merely necessary to have signal intensities from these calibration zones corresponding to two points on a standard curve such that the instrument can calculate the amount of analyte in the test sample by comparison of the calibration zones, one low and one high and with the standard curve data contained within the instrument's data system.

The cartridge is taken into the instrument, the test read by comparison with the respective signal intensity of each calibration zone and a result calculated. The instrument can be a stand alone instrument, which prints out or displays a result for the operator, or it can be connected to the laboratory's central computer and aligned with other patient data. The results obtained in this way are fully quantitative and such an instrument is valuable in monitoring a patient's analyte levels during therapy or for following the course of disease or progress after surgery.

EXAMPLE 5

Quantitative Determination of D-dimer by instrument reading

A comparison of a quantitative determination of D-dimer by instrument reading with D-dimer concentration as determined by enzyme immunoassay for D-dimer is shown in FIG. 12. The results are presented as a linear regression analysis, thus, indicating a strong correlation between instrument reading and EIA. This instrument reading comparison was made with 187 plasma samples of clinical origin using 25 µL of sample. At ten minutes after the tests were run the reflectance signal of the test zone was compared with the reflectance signal of the calibration zones. The instrument readings were performed using a simple reflectance instrument (Tobias IQ 150 Portable Computing Reflection Desitometer, Tobias Associates Inc, Ivyland, PA, USA).

EXAMPLE 6

Quantitative determination of myoglobin by instrument reading

A comparison of a quantitative determination of myoglobin by instrument reading with the myoglobin concentration as determined by enzyme immunoassay for myoglobin is shown in FIG. 13. The results are presented as a linear regression analysis, thus, indicating a strong correlation between instrument reading and EIA. This instrument reading comparison was made with 127 plasma samples of clinical origin using 25 µL of sample. At ten minutes after the tests were run the reflectance signal

of the test zone was compared with the reflectance signal of the calibration zones. The instrument readings were performed using the reflectance instrument referred to in Example 6.

EXAMPLE 7

Adjustment of calibrator line intensity

A series of test strips comprising the lateral flow liquid permeable medium of the invention are run containing a fixed concentration of calibration agent, *e.g.* streptavidin-gold with the strips coated at the calibration zone with a fixed BSA concentration but with a variable biotin content varying from 0.1 to 3.0 moles of biotin per mole of BSA.

Using the reflectance instrument described above, the reflectance signal at the calibration zone is determined (FIG. 14). The reflectance signal is plotted against the concentration of biotin-BSA and is used as a standard curve as follows:

For a given target analyte, a small number of samples with a known analyte concentration are run on strips containing target detection agent and target capture agent. The reflectance signal at the capture zone is then read off the standard curve of reflectance signal versus biotin-BSA concentration to determine the concentration of the calibration capture (biotin-BSA) which is required to match a particular clinical cut-off value (Table 3). This is of particular use in the production of tests in which a semi-quantitative determination of analyte concentration is required. For an instrument read test, the calibrator concentrations do not necessarily have to match a particular clinical cut-off value, hence using the calibrator standard curve, calibrators can be chosen which cover the dynamic range of the target analyte which is determined as described above using the reflectance signals of samples with known values run on tests containing target analyte detection and calibration agents.

BSA is biotinylated using NHS-LC-Biotin in carbonate buffer to yield 25 moles of biotin per mole of BSA. This stock solution of biotin-BSA

is then diluted in BSA to the required concentration.

In another embodiment of the invention the labelled analyte detection agent and labelled calibration agent may be applied to the lateral flow liquid permeable medium in solution and, thus, these reagents may be included with the test sample if desired.

Significant advantages of the present invention in comparison with the prior art discussed above include the following:

(a) a simple single-step assay is provided which is self contained and requires only the addition of the test sample;

(b) the use of a single-step assay greatly enhances the reproducibility and convenience of the assay;

(c) the affinity and avidity of the calibration zone reactants which may be used are significantly greater than the test zone reactants which substantially eliminates interference and facilitates quantitation of a wide range of analytes as described above. For example, the calibration zone reactions suitably have an affinity constant of 10^{-15}M which is six orders of magnitude higher affinity than normal antibody affinities;

(d) the same calibration pair can be used for a wide range of target analytes with minimal experimentation and effort thereby avoiding excessive calibration for each assay which is of significant commercial advantage; and

(e) the assay is essentially continuous in operation and thus avoids discontinuity which is a prerequisite of multiple step assays.

TABLES

TABLE 1 Semi-quantitative determination of D-dimer effected by comparison of respective signals at the test and calibration zones

TEST RESULT		ASSIGNED VALUE
1.	No visible signal at the test zone	Less than 120 ng/mL
2.	A signal intensity at the test zone that is less than or equal to that of the weakest calibration zone	Less than 120 ng/mL
3.	A signal intensity at the test zone that is between the intensity of the two calibration zones	Greater than 120 ng/mL but less than 1000 ng/mL
4.	A signal intensity at the test zone that is greater than that of the strongest calibration zone	Greater than 1000 ng/mL

TABLE 2 Semi-quantitative determination of digoxin effected by comparison of respective signals at the test and calibration zones

	TEST RESULT	ASSIGNED VALUE
1.	A signal intensity at the test zone that is stronger than either of the calibration zones	Less than 0.8 ng/mL
2.	A signal intensity at the test zone that is between the intensity of the calibration zones	Greater than 0.8 ng/mL but less than 1.6 ng/mL
3.	A signal intensity at the test zone that is less than that of the weakest calibration zone	Greater 1.6 ng/mL

TABLE 3 Calibration receptor concentrations for several different analytes with respective cut-off levels

Analyte	Calibrator Concentration	Cut-off Value	Reflectance Signal of Cut-off Value
Myoglobin	0.5 moles biotin/mole BSA	70 ng/mL	640
D-dimer	0.35 moles biotin/mole BSA	120 ng/mL	550
C-Reactive Protein	0.225 moles biotin/mole BSA	10 µg/mL	250
Canine Heartworm	0.25 moles biotin/mole BSA	100 ng/mL	275

LEGENDS

FIG. 6

Comparison of D-dimer EIA vs Quantitative Determination of D-dimer by Instrument.

FIG. 7

Comparison of the Quantitative Measurement of Myoglobin by EIA versus Visual Semi-Quantitative Determination of Myoglobin.

FIG. 12

Comparison of D-dimer EIA vs Quantitative Determination of D-dimer by Instrument.

FIG. 13

Comparison of Myoglobin EIA vs Quantitative Determination of Myoglobin by Instrument.

FIG. 14

Standard Curve of Biotin-BSA Concentration vs Reflectance Signal.

CLAIMS

1. A method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium an analyte receptor capable of binding to the target analyte;

(ii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element an analyte detection agent which detects the presence of target analyte in the test sample, said analyte detection agent having a label associated therewith;

(iii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element a calibration agent having a label associated therewith;

(iv) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(v) contacting the lateral flow liquid permeable medium with the test sample; and

(vi) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

2. A method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium a predetermined amount of analyte;

(ii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element an analyte detection agent which detects the target analyte if present in the test sample, said analyte detection agent having a label associated therewith;

(iii) diffusibly attaching to a support medium which may comprise the lateral flow liquid permeable medium or a separate support element a calibration agent having a label associated therewith;

(iv) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(v) contacting the lateral flow liquid permeable medium with the test sample; and

(vi) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

3. A method as claimed in Claim 1 wherein the analyte detection agent is a predetermined amount of the target analyte having a label associated therewith.

4. A method as claimed in Claim 1 or Claim 2 wherein the label is a direct visual label selected from the group including a colloidal metallic or non-metallic particle, a dye particle, an enzyme, a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance.

5. A method as claimed in Claim 4 wherein the direct visual label is a colloidal metallic particle.

6. A method as claimed in Claim 5 wherein the colloidal metallic particle is colloidal gold.

7. A method as claimed in Claim 1 or Claim 2 wherein the calibration agent is selected from the group including avidin or streptavidin and the calibration receptor is biotin.

8. A method as claimed in Claim 1 or Claim 2 wherein the calibration receptor is avidin or streptavidin and the calibration agent is biotin.

9. A method as claimed in Claim 1 or Claim 2 wherein the step of comparing signals associated with each label is effected by a signal

measuring instrument to provide quantitative determination of the target analyte in the test sample.

10. A method as claimed in Claim 1 or Claim 2 further including a filtering step for filtering the test sample.

11. A method as claimed in Claim 1 or Claim 2 further including a procedural control step which indicates validation of the method.

12. A method as claimed in Claim 1 or 2 wherein the analyte detection agent and the calibration agent have the same label associated therewith.

13. A lateral flow liquid permeable medium adapted to convey fluids in at least one fluid flow direction for use in the method according to Claim 1, said liquid permeable medium comprising:

(1) at least one test zone having non-diffusibly attached therein an analyte receptor which is capable of participating in the formation of an immobilized specific binding pair with an analyte or analyte detection agent;

(2) at least one calibration zone having non-diffusibly attached therein a predetermined amount of a calibration agent receptor which is capable of participating in the formation of an immobilized specific binding pair with a calibration agent; and

(3) an assay reagent zone including a calibration agent having a label associated therewith and the analyte detection agent which a label associated therewith.

14. A lateral flow liquid permeable medium adapted to convey fluids in at least one fluid flow direction for use in the method according to Claim 2, said liquid permeable medium comprising:

(1) at least one test zone having non-diffusibly attached therein a predetermined amount of analyte which is capable of participating in the formation of an immobilized specific binding pair with an analyte detection agent;

(2) at least one calibration zone having non-diffusibly attached therein a predetermined amount of a calibration agent receptor which is

capable of participating in the formation of an immobilized specific binding pair with a calibration agent; and

(3) an assay reagent zone including a calibration agent having a label associated therewith and the analyte detection agent also having a label associated therewith.

15. A lateral flow liquid permeable medium as claimed in Claim 13 or Claim 14 wherein the assay reagent zone is provided by a separate support element in fluid communication with the lateral flow liquid permeable medium.

16. A lateral flow liquid permeable medium as claimed in Claim 13 or Claim 14 further including a filter in liquid communication therewith.

17. A lateral flow liquid permeable medium as claimed in Claim 13 or Claim 14 further including a procedural control zone.

18. A lateral flow liquid permeable medium as claimed in Claim 13 or Claim 14 further including an absorbent pad which is downstream of said test and said at least one calibration zone, which absorbent pad is adapted to absorb an excess amount of a test sample or a reagent solution.

19. A housing enclosing the lateral flow membrane of Claim 13 or Claim 14 having windows for viewing of the or each test zone and the or each calibration zone.

20. A housing as claimed in Claim 19 having a further window for viewing of the procedural control zone.

21. A housing as claimed in Claim 19 having an aperture for introduction of the target analyte.

22. A signal measuring instrument for use with the method of Claim 1 or Claim 2 for comparison of signals associated with each label at the test and calibration zone(s) to effect determination of the target analyte in the test sample.

23. A signal measuring instrument as claimed in Claim 22 incorporating the housing of any one of Claims 19-21.

24. A method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium an analyte receptor capable of binding to the target analyte;

(ii) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(iii) contacting the lateral flow liquid medium with (a) an analyte detection agent which detects the presence of target analyte in the test sample and (b) a calibration agent, each of said analyte detection agent and said calibration agent having a label associated therewith;

(iv) contacting the lateral flow liquid permeable medium with the test sample; and

(v) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

25. A method for quantitative or semi-quantitative determination of target analyte(s) in a test sample, said method comprising the steps of:

(i) non-diffusibly attaching to at least one test zone of a lateral flow liquid permeable medium a predetermined amount of analyte;

(ii) non-diffusibly attaching to at least one calibration zone of the lateral flow liquid permeable medium a calibration agent receptor capable of binding the calibration agent;

(iii) contacting the lateral flow liquid medium with (a) an analyte detection agent which detects the presence of target analyte in the test sample and (b) a calibration agent, each of said analyte detection agent and said calibration agent having a label associated therewith;

(iv) contacting the lateral flow liquid permeable medium with the test sample; and

(v) comparing signals associated with each label at the test zone(s) and calibration zone(s) to effect determination of the target analyte in the test sample.

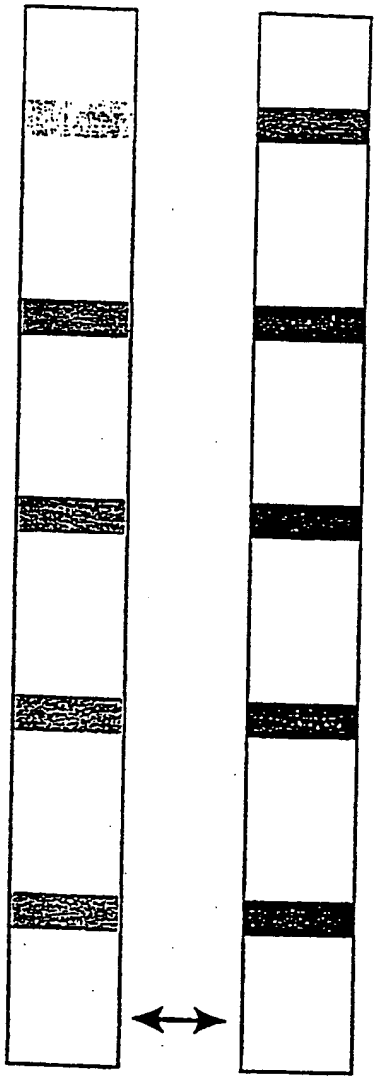


FIG. 1

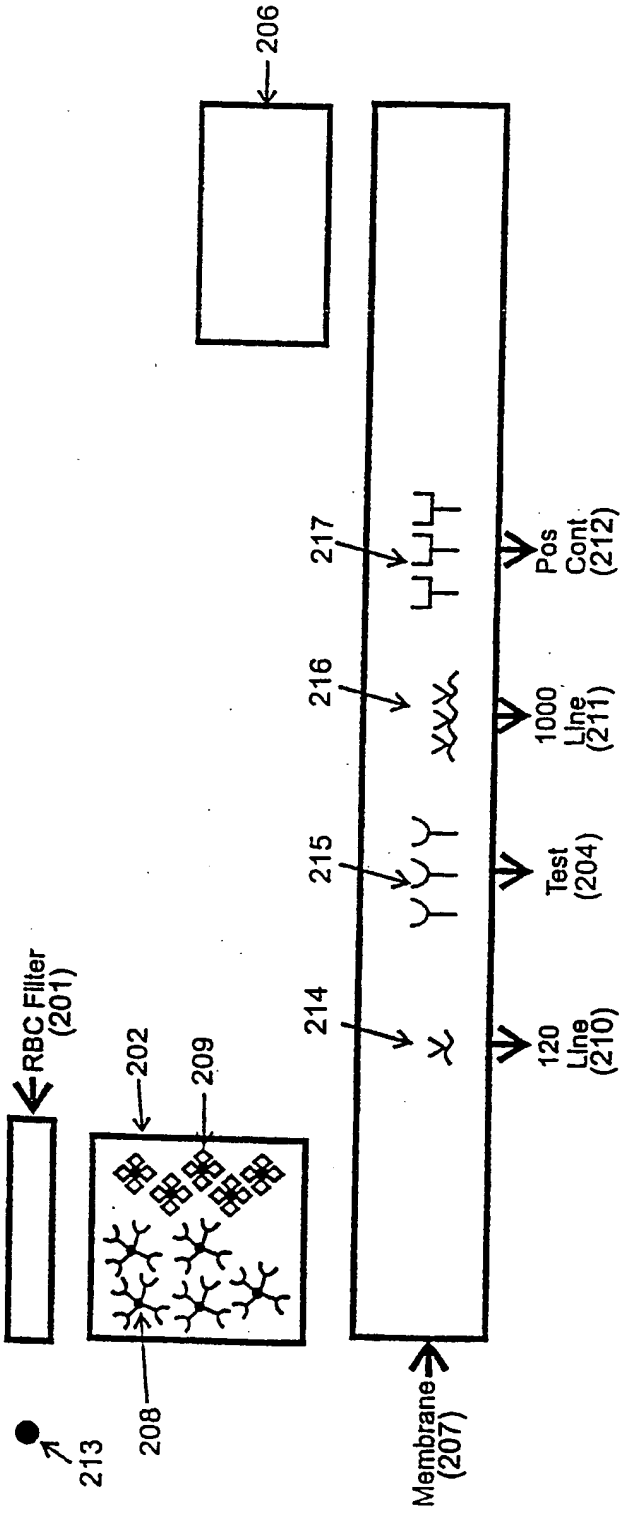
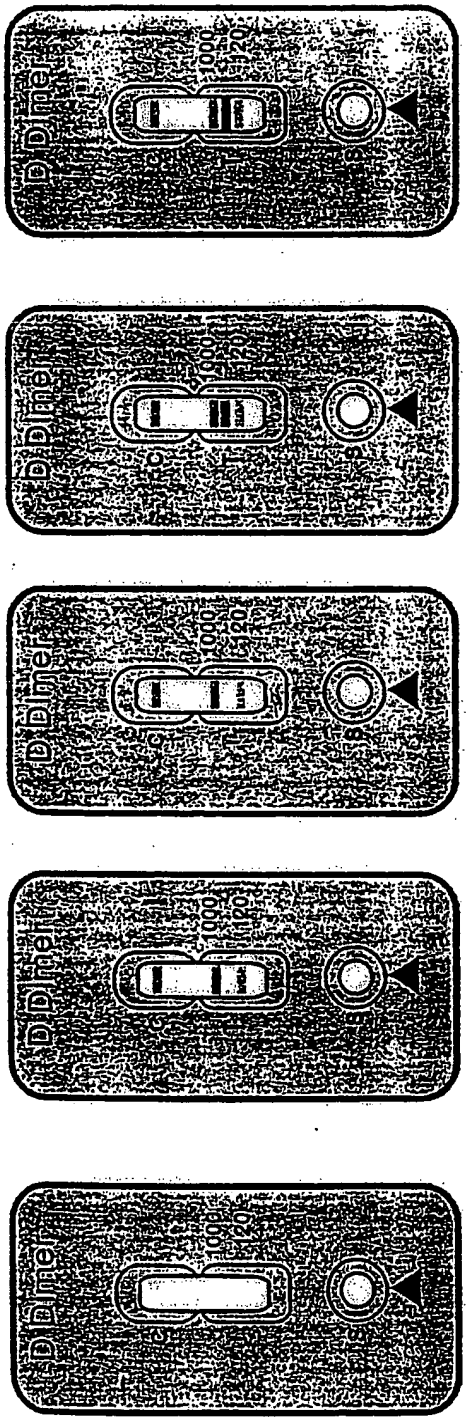

206
217
Pos Cont (212)
216
1000 Line (211)
215
Test (204)
214
120 Line (210)
RBC Filter (201)
202
209
208
213
Membrane (207)

FIG. 2



Unused Device — FIG. 3a

<120ng/mL — FIG. 3b

<120ng/mL — FIG. 3c

>120ng/mL <1000ng/mL — FIG. 3d

>1000ng/mL — FIG. 3e

FIG. 3

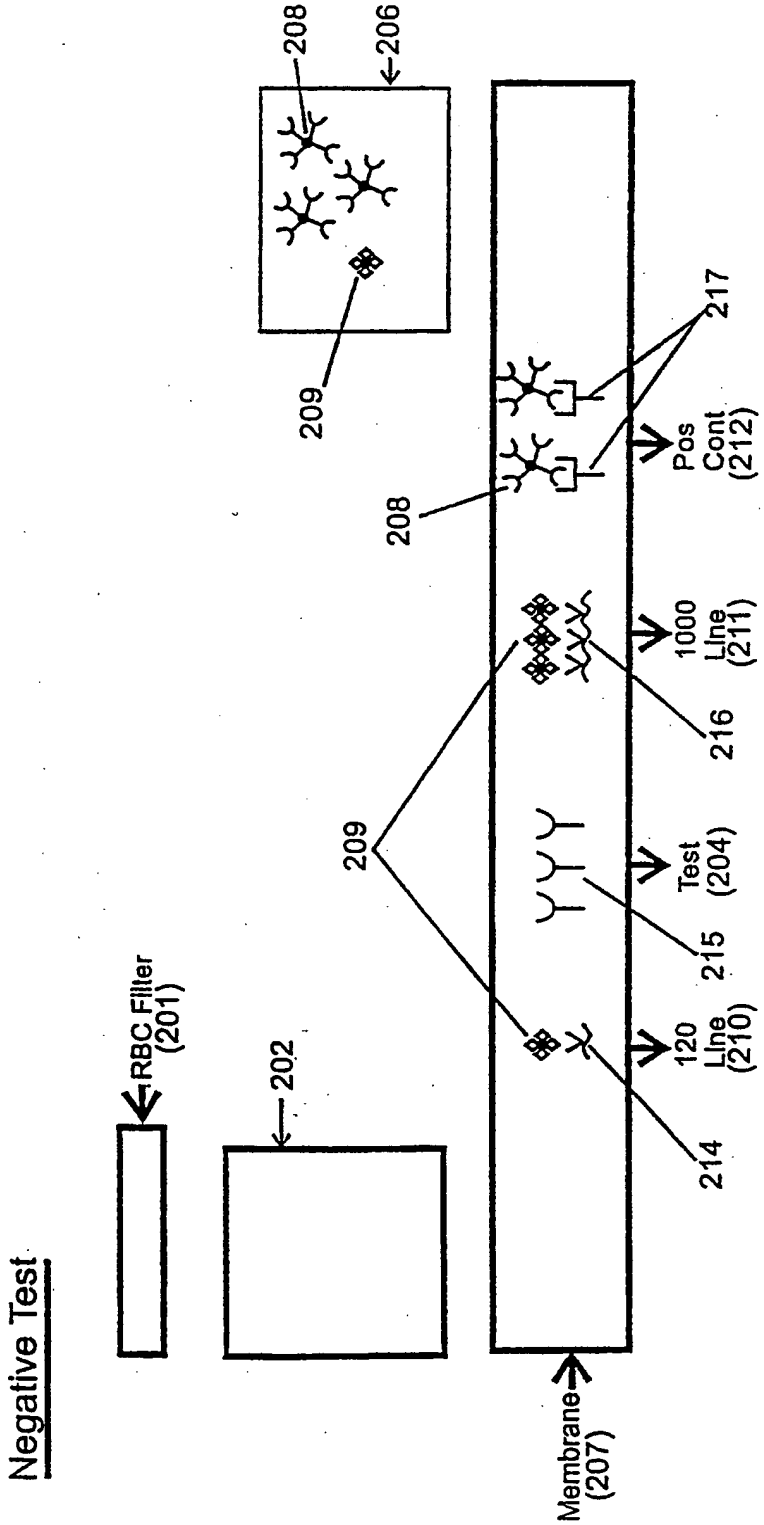

Negative Test
RBC Filter (201)
202
Membrane (207)
208
206
209
217
Pos Cont (212)
208
1000 Line (211)
216
209
Test (204)
215
120 Line (210)
214

FIG. 4

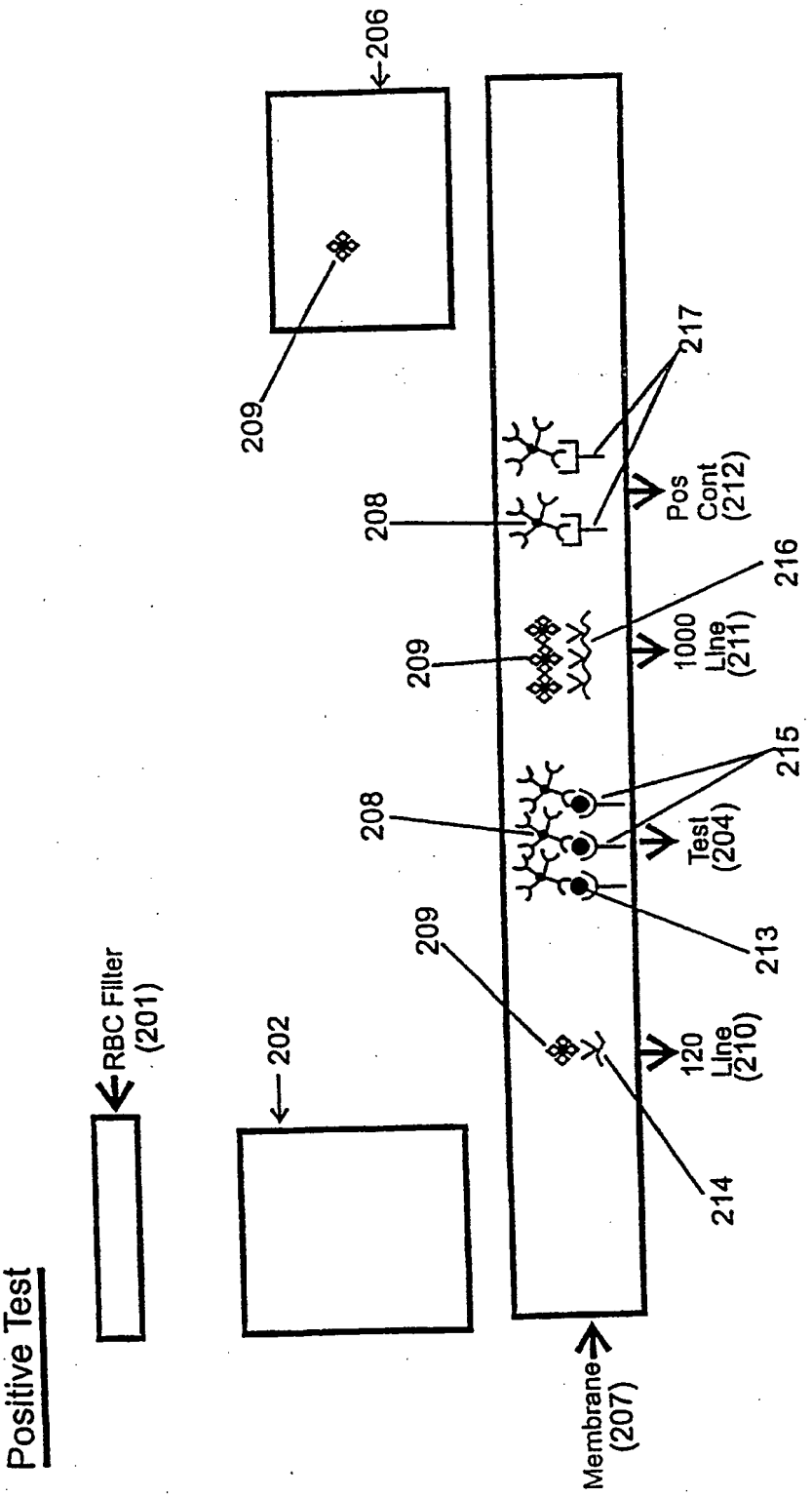

Positive Test
RBC Filter (201)
202
206
209
208
209
208
209
217
Pos Cont (212)
216
1000 Line (211)
215
Test (204)
213
120 Line (210)
214
Membrane (207)

FIG. 5

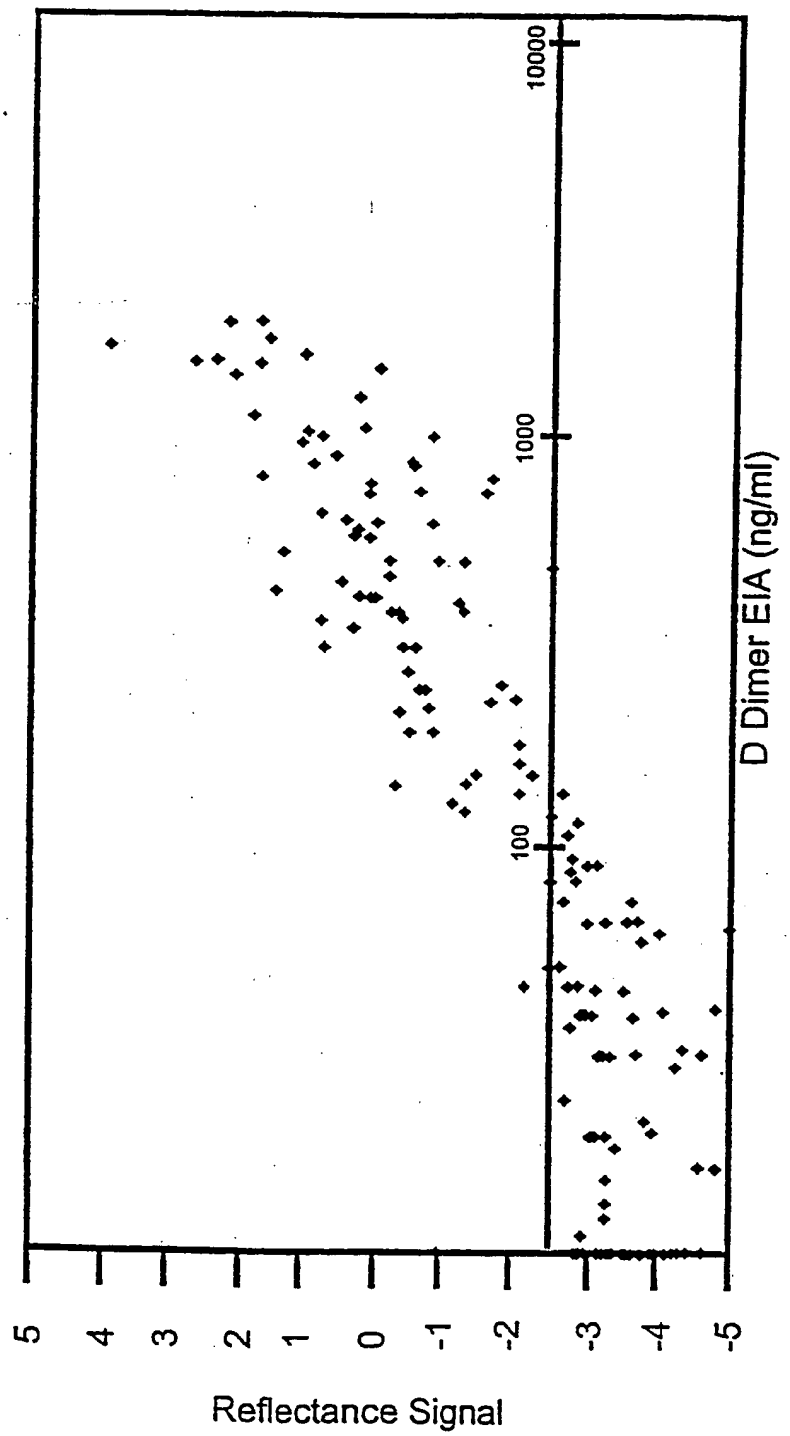
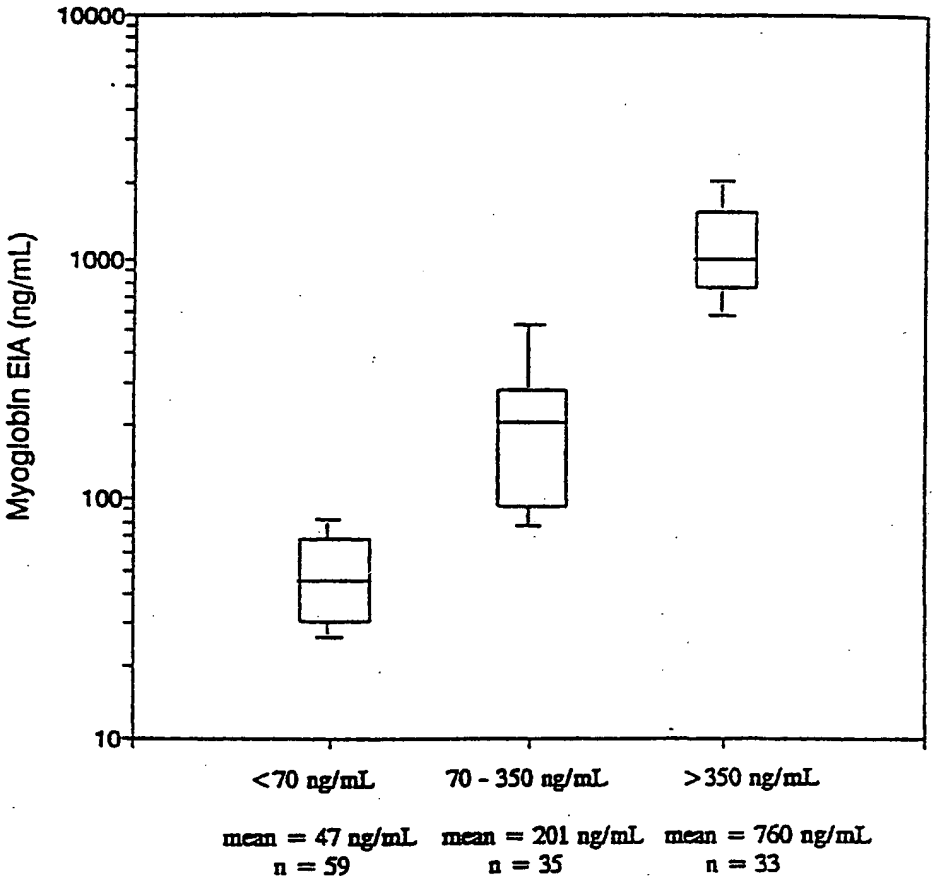

10000
1000
100
D Dimer EIA (ng/ml)
5
4
3
2
1
0
-1
-2
-3
-4
-5
Reflectance Signal

FIG. 6

FIG. 7


10000
1000
100
10
Myoglobin EIA (ng/mL)
<70 ng/mL
70 - 350 ng/mL
>350 ng/mL
mean = 47 ng/mL
n = 59
mean = 201 ng/mL
n = 35
mean = 760 ng/mL
n = 33

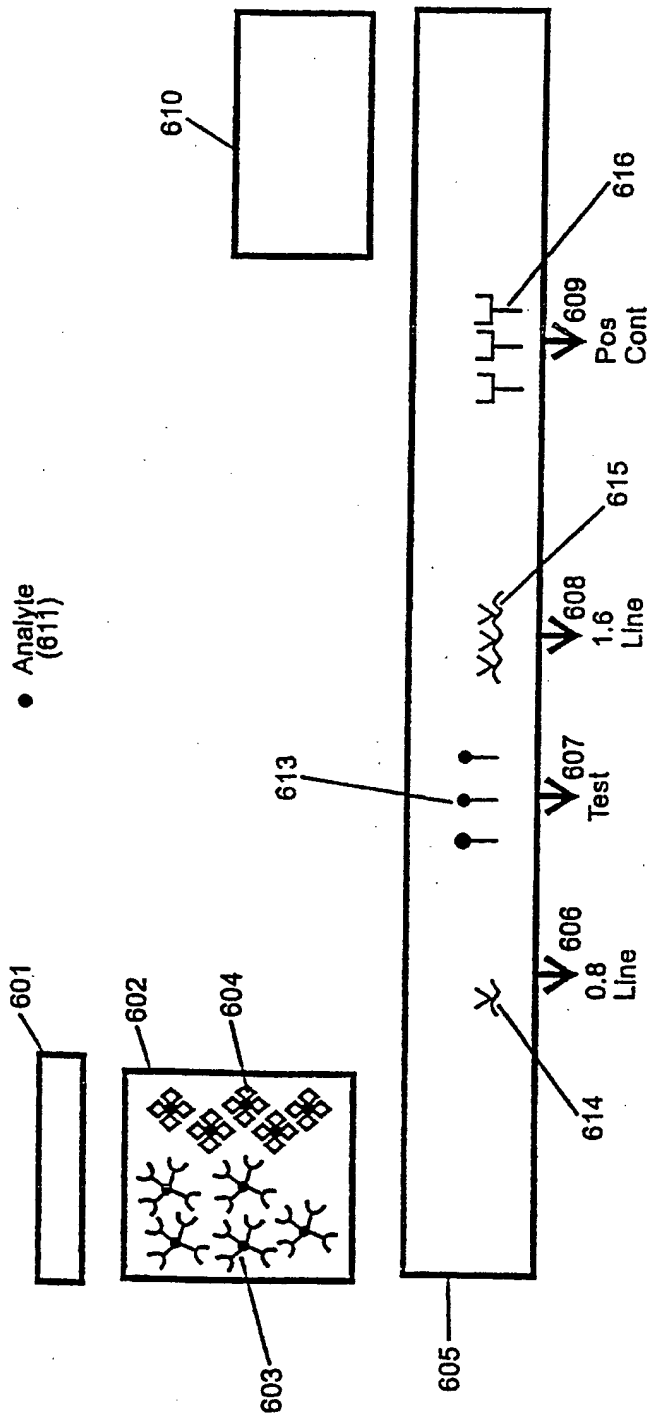

610
616
609
Pos
Cont
615
608
1.6
Line
Analyte
(611)
613
607
Test
606
0.8
Line
601
602
604
614
603
605

FIG 8

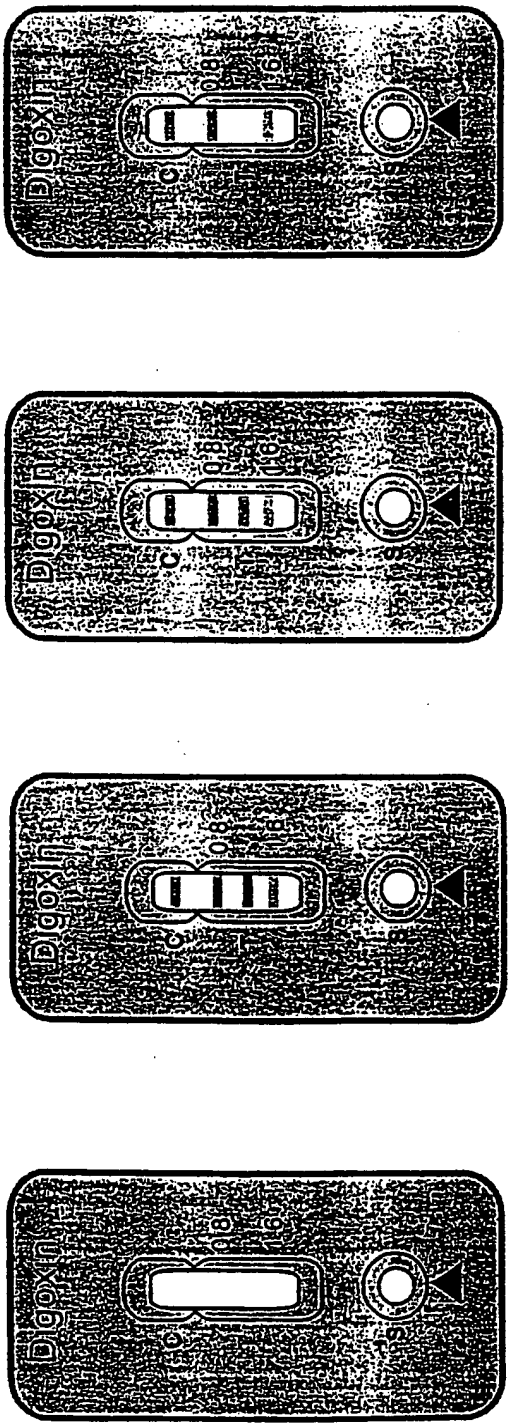


FIG. 9a FIG. 9b FIG. 9c FIG. 9d

FIG. 9

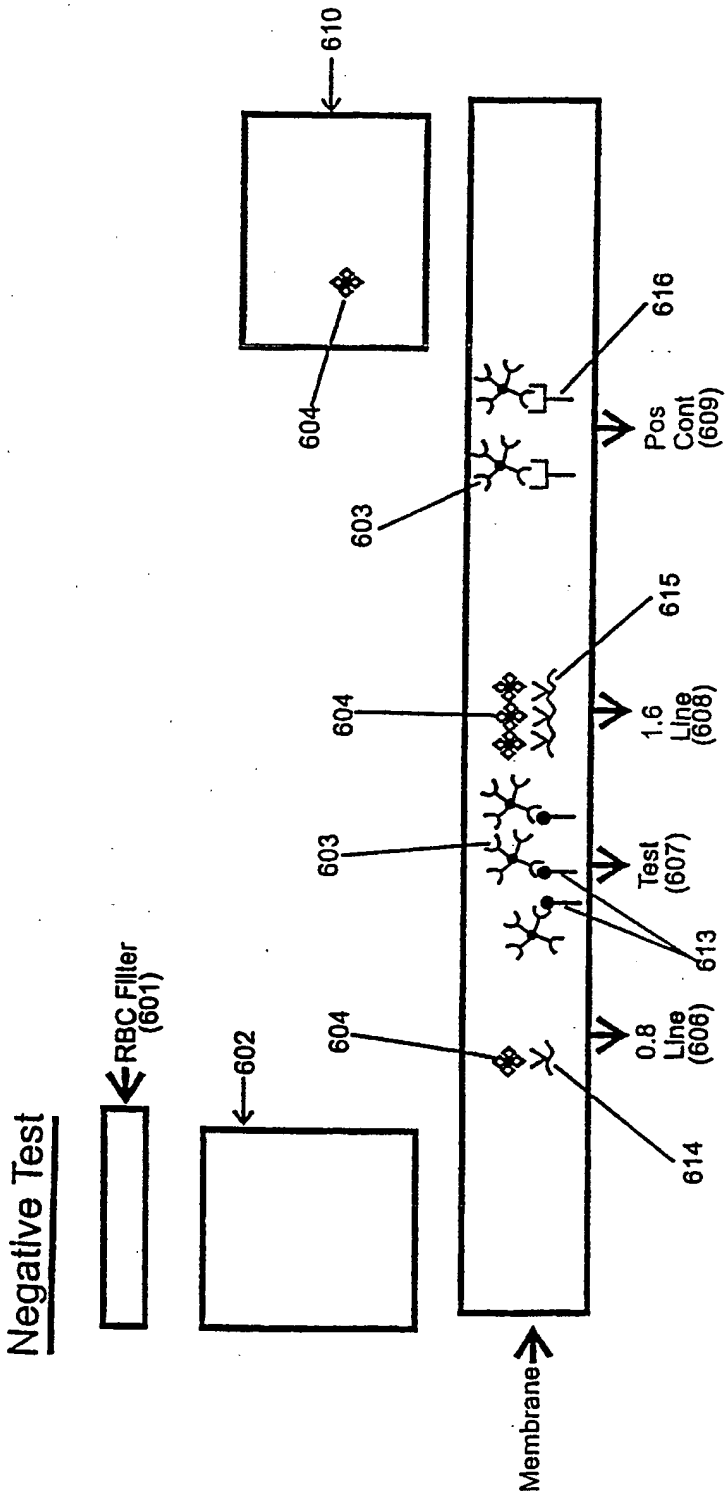

Negative Test
RBC Filter (601)
602
610
604
603
616
Pos Cont (609)
615
1.6 Line (608)
Test (607)
613
0.8 Line (606)
614
Membrane

FIG. 10

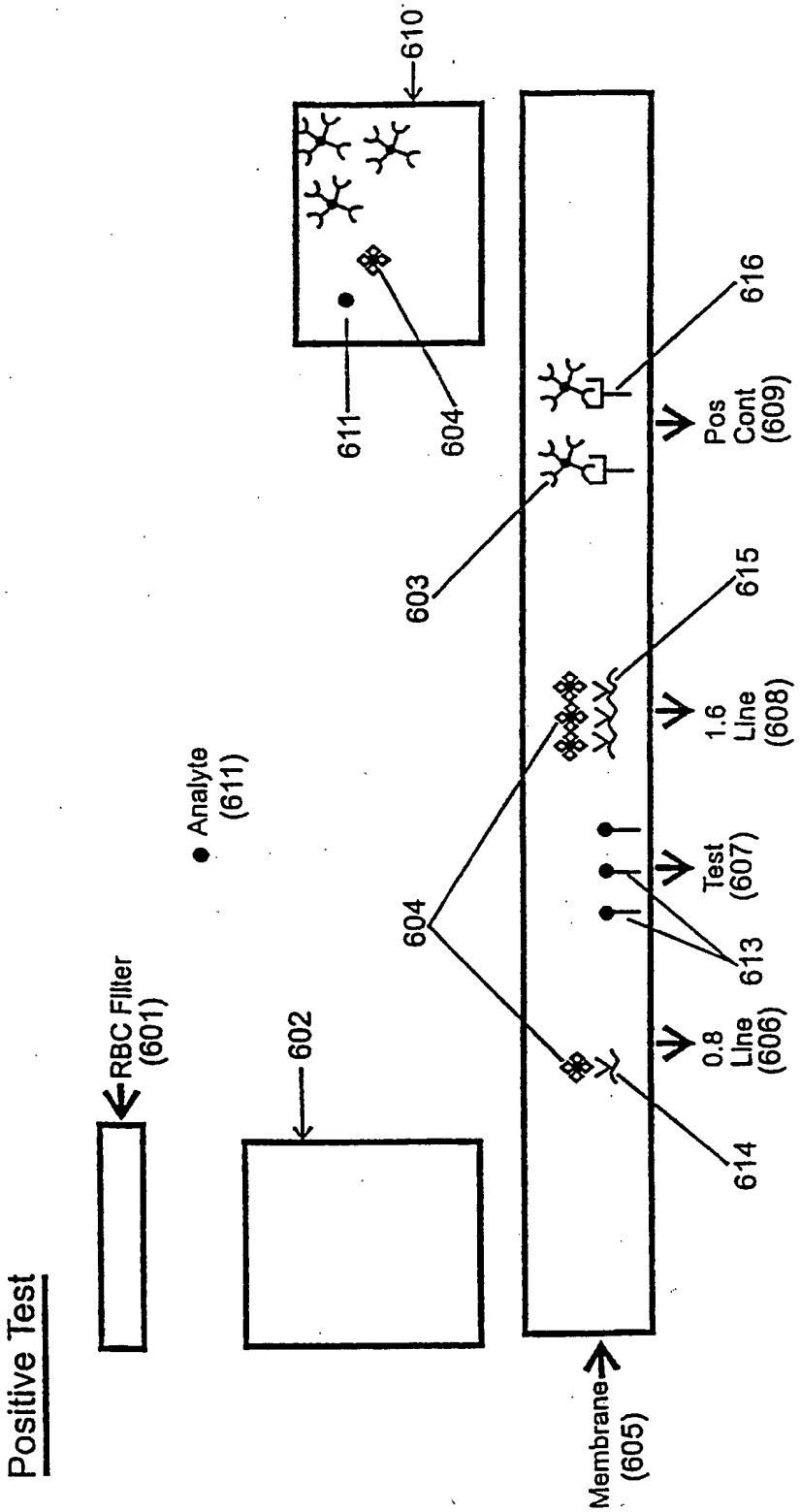

Positive Test
RBC Filter (601)
602
Analyte (611)
610
611
604
603
616
Pos Cont (609)
615
1.6 Line (608)
Test (607)
613
604
0.8 Line (606)
614
Membrane (605)

FIG. 11

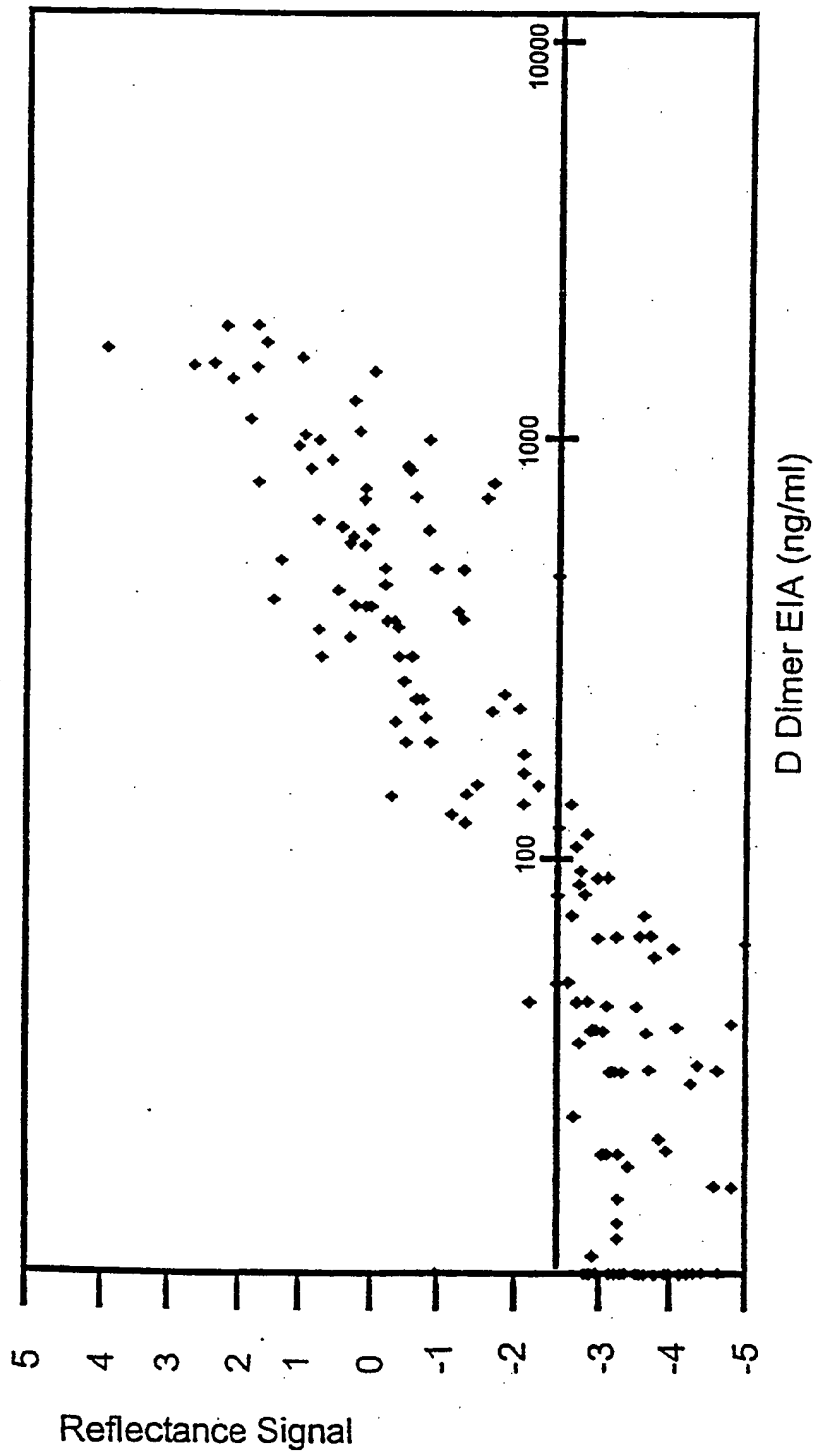

10000
1000
100
D Dimer EIA (ng/ml)
5
4
3
2
1
0
-1
-2
-3
-4
-5
Reflectance Signal

FIG. 12

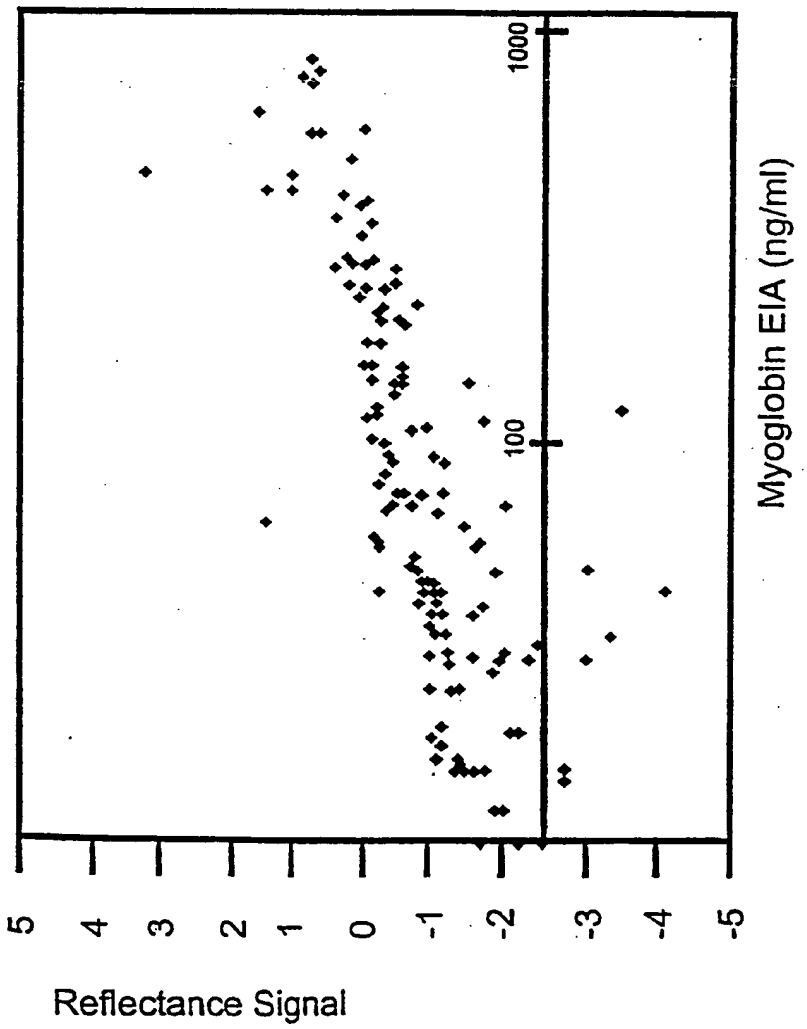
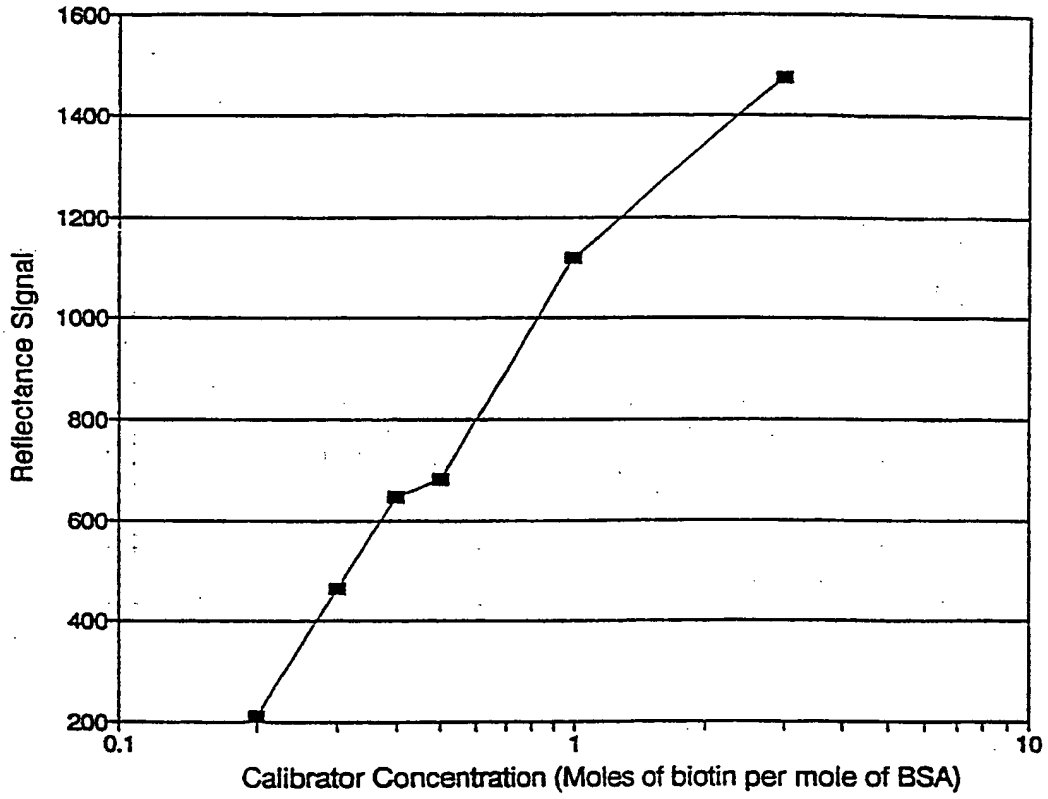

1000
100
Myoglobin EIA (ng/ml)
5
4
3
2
1
0
-1
-2
-3
-4
-5
Reflectance Signal

FIG. 13

FIG. 14


1600
1400
1200
1000
800
600
400
200
Reflectance Signal
0.1
1
10
Calibrator Concentration (Moles of biotin per mole of BSA)

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00557

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/577, 33/566, 33/545, 33/548, 33/551

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: G01N 33/- with keywords as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT ORBIT, STN (IMMUNO:, ELISA, QUANTITATIVE, QUANTIFY, CALBRAT: STANDARD:, REFERENCE)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU, A, 77274/91 (HYBRITECH INCORPORATED) 26 September 1991	1-25
A	FR, A1,2702841 (TOLEDANO J) 23 September 1994	1-25
A	WO, A, 93/15230 (ABBOTT LABORATORIES) 05 August 1993	1-25

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1996	7 JAN 1997
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **DEBORAH LALLY** Telephone No.: (06) 283 2533

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INTERNATIONAL SEARCH REPORT

ernational Application No.
PCT/AU 96/00557

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AU, A, 41702/89 (ABBOTT LABORATORIES) 10 May 1990	1-25
A	EP , A, 325449 (IDEXX CORPORATION) 26 July 1989	1-25
A	US, A, 4868108 (HYGEIA SCIENCES INC) 19 September 1989.	1-25
A	EP, A, 342586 (ENZO BIOCHEM INC) 23 November 1989	1-25
A	AU, A, 79031/87 (ABBOTT LABORATORIES) 31 March 1988	1-25
A	Therapeutic Drug Monitoring, Vol 9, No 2, 1987, pages 190-6, Opheim, K. et al; "Calibration, quality control, and stability of a quantitative enzyme immunochromatographic method for therapeutic drug monitoring".	1-25
A	Thrombosis and Haemostasis, Vol 68, No 3, 1992, pages 273-7, Nieuwenhuizen, W. et al "Rapid monoclonal antibody-based enzyme immunoassay (EIA) for the quantitative determination of soluble fibrin in plasma"	1-25
P,A	Clinical Chemistry, Vol 42, No 4, 1996, pages 545-550, Espana F et al "Quantitative immunoassay for complexes of prostate-specific antigen with α_2-macroglobulin".	1-25

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00557

Information on patent family members

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	77274/91	AU	77274/91	AT	76689	AU	70447/87
		CA	1286987	DE	3779365	EP	253464
		ES	2042550	JP	62231168		
WO	93/15230	AU	34396/93	EP	643777	JP	7503540
		WO	9315230	WO	9412928	DE	69303725
		EP	670061	JP	8504525	US	5343417
AU	41702/89	AT	78597	AT	83325	AU	63502/86
		AU	41702/89	CA	1281642	CA	1301648
		DE	3686116	DE	3687276	EP	217403
		EP	389003	ES	2001811	GR	862328
		JP	62228167	JP	5180841	JP	5088785
		JP	6050973	JP	2514878	KR	9402520
		US	5008080	US	5149622	US	5160701
		AT	116443	AU	31564/89	CA	1332807
		DE	68920176	EP	335244	ES	2068844
		JP	1299464	KR	9209420		
EP	325449	AT	120008	DE	68921650	EP	325449
		JP	1245157	US	5356785		
EP	342586	DE	68924802	EP	342586	JP	2027976
AU	79031/87	AT	85130	AU	79031/87	CA	1303493
		DE	3783845	EP	262328	ES	2038973
		JP	63096559	JP	7036017	US	4960691
		US	5310650	AU	51196/90	CA	2012355
		EP	387696	JP	2283299		

END OF ANNEX